Phosphatidylserine-Specific Receptor Contributes to TGF-β Production in Macrophages through a MAP Kinase, ERK

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We explored the involvement of the phosphatidylserine (PS)-receptor in the production of TGF-β by macrophages treated with PS-liposomes. The binding of anti-PS-receptor antibody to macrophages was specifically inhibited by PS-liposomes. The antibody led to an increase in the production of TGF-β, and also activated ERK, a member of the MAP kinase. But no activations in p38 and JNK were observed. ERK inhibitor, U0126 completely prevented TGF-β production. On the addition of a TGF-β neutralizing antibody or U0126, the inhibitory effect of the anti-PS-receptor antibody on macrophage function, nitric oxide production, was restored. These findings suggested that TGF-β is one of factors produced by PS-liposomes, and the ERK signaling pathway via the PS-receptor is intimately involved in the production of TGF-β in macrophages.

Key words macrophage; nitric oxide; phosphatidylserine (PS)-receptor; PS-liposome; TGF-β, MAP kinase

Nitric oxide (NO) is a reactive free radical gas synthesized in mammalian tissues from l-arginine and molecular O₂ by NO synthase (NOS).1,2) Macrophages express an inducible form of NOS (i-NOS) in response to cytokines or endotoxins such as lipopolysaccharide (LPS). NO produced by i-NOS is an important mediator of acute or chronic inflammation, and also contributes to the killing of virally infected cells, tumor cells and some pathogens.3–5) On the other hand, the production of NO by i-NOS is regulated by the amount of NOS whose production is transcriptionally regulated are involved in diseases such as septicemia and rheumatoid disease. Thus, NO produced by i-NOS appears to be either protective or deleterious, depending on its local milieu.6–8)

When liposomes are injected intravenously, they are rapidly removed from the blood circulation and taken up by macrophages in the reticuloendothelial system such as the liver and spleen.9,10) The interaction of liposomes with macrophages has been studied, and liposomal properties such as charge, size, and lipid composition have been shown to affect liposomal uptake by macrophages.11–13)

In our recent studies, negatively charged liposomes composed of phosphatidylserine (PS-liposomes) inhibited production of NO in thioglycollate-elicited mouse peritoneal macrophages stimulated with LPS and this inhibition was implicated in the production of TGF-β.14–16) To clarify this inhibitory effect of PS-liposomes on macrophage functions, we focused on TGF-β, a multifunctional polypeptide growth factor that has been increasingly recognized as an important immunoregulatory molecule, although its reported effects on immunological responses are often contradictory.17,18) Our recent findings indicated that macrophages produced TGF-β following treatment with PS-liposomes and the production of TGF-β was attributable to the activation of ERK, a MAP kinase.19) However, it remains to be elucidated how macrophages recognize PS-liposomes and secrete TGF-β.

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Material and methods

Materials Phosphatidylcholine (PC) from egg yolk was purchased from Nippon Oil and Fat Co., Ltd. (Tokyo, Japan). Phosphatidylserine (PS) from bovine brain, lipopolysaccharide (LPS) from Escherichia coli (serotype 0111; B4), anti-phosphatidylserine receptor antibody, and FITC-conjugated anti-rabbit IgG antibody were obtained from Sigma Co., Ltd. (St. Louis, MO, U.S.A.). Mouse monoclonal anti-TGF-β1, -β2, and -β3 antibodies were from GT (Minneapolis, MN, U.S.A.).

Preparation of Liposomes Multilamellar liposomes were prepared by vortexing and passed through a membrane filter (0.45 μm; Corning Glassworks, Corning, NY, U.S.A.) before use. Lipid compositions of liposomes were PS : PC : cholesterol = 2 : 1 : 1 (PS-liposomes).

Preparation of Macrophages C3H/HeN mice (male, 6–8 weeks old) were purchased from Japan SLC Inc., (Shizuoka, Japan). Animal use and relevant experimental procedures were approved by the Tokyo University of Pharmacy and Life Science Committee on Care and Use of Laboratory Animals. C3H/HeN mice were injected intraperitoneally with 1.0 ml of 3% thioglycollate (Difco Laboratory, Detroit, MI, U.S.A.). On day 4, the peritoneal macrophages were prepared according to our previous method.14)

FACS Analysis Macrophages (×10⁶ cells) suspended in RPMI 1640 medium supplemented with 10% FCS were treated with liposomes. After three washes with PBS, cells were resuspended in PBS in the presence of anti-phos-
phatidylserine receptor antibody (150 μg/ml) for 30 min, at 4 °C. Moreover, after three washes with PBS, cells were resuspended in PBS in the presence of FITC-conjugated anti-rabbit IgG antibody (50 μg/ml) for 30 min at 4 °C. Finally, after three washes with PBS, cells were resuspended in 1 ml of PBS and the PS-specific receptor expression or binding of liposomes to the PS-specific receptor was evaluated by flow cytometry (FACSCalibur, Becton-Dickinson).

Nitrite Assay Macrophages (1×10⁵ cells/well) were treated with anti-phosphatidylserine receptor antibody at specific concentrations, and then further incubated for 48 h with LPS (10 μg/ml). NO production was estimated by measuring nitrite in the culture supernatant using Griess reagent as described by Stuehr and Nathan.²⁷ In brief, an aliquot of the culture supernatant was mixed with an equal volume of 1% sulfanilamide and 0.1% N-1-naphthylenediamine dihydrochloride in 5% phosphoric acid. The absorbance was determined at 540 nm. Sodium nitrite, diluted in HBSS at concentrations of 0—100 μM, was used to generate a standard curve.

RT-PCR Analysis TGF-β gene expression was detected by RT-PCR. Total RNA (2 μg) was isolated from macrophages with Isogen solution (Nippon Gene, Toyama, Japan). cDNA was synthesized by SuperScript II. Then, cDNAs were amplified with specific primers for TGF-β. β-Actin primers were used as an internal control. Primers were designed based on the mouse sequences (TGF-β1; forward, 5'-TTGGCATAGAGGTCTTTACGGA-3', reverse, 5'-CAGGAGCGCACAAT-CATGTT-3', β-actin; forward, 5'-GCACCCACCT-CTCAATGAG-3', reverse, 5'-TTGCATAGGTCCTTTACGGA-3'). PCR was performed for 28 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 60 s, and extension at 72 °C for 60 s. The amplified products were analyzed on 1.5% agarose gels containing 0.1 μg/ml of ethidium bromide.

Detection of TGF-β Macrophages (5×10⁵ cells/well) were treated with anti-PS-receptor antibody (20 μg/ml) for a specific period. Supernatants were collected from cell cultures and frozen at −80 °C prior to use. TGF-β concentrations were determined by ELISA using pairs of purified capture and biotinylated detection antibodies recognizing murine TGF-β, respectively, according to the manufacturer’s directions (BD Biosciences).

Western Blotting Analysis Macrophages (1×10⁶ cells/well) were treated with anti-PS-receptor antibody (20 μg/ml) for given periods. Cells were then lysed as described.¹⁵ For the determination of ERK activation, cell lysates were separated by 12% SDS-PAGE, blotted on Immobilon P membranes (Nihon Millipore, Tokyo, Japan) and analyzed using a Phospho plus p44/p42 MAP Kinase (Thr202/Tyr204) Antibody Kit.

RESULTS AND DISCUSSION

Expression of PS-Receptor and Binding of PS-Liposomes As noted above, recently, we demonstrated that PS-liposomes inhibited production of NO in thio glycollate-elicited mouse peritoneal macrophages stimulated with LPS and TGF-β is one of factors produced by PS-liposomes that suppresses production of NO in macrophages. However, it remains to be elucidated which receptor is predominantly involved in the recognition of PS-liposomes, and consequently contributes to the inhibition of NO production. We have already reported that CD68 and CD14 did not contribute to the inhibitory effect of PS-liposomes on the production of NO induced by LPS.²⁸,²⁹ In this paper, we thus focused on the PS-receptor, and examined the binding of antibody to mouse peritoneal macrophages by flow cytometry. As shown in Fig. 1, the intensity of fluorescence shifted to an upper field in the presence of anti-PS-receptor antibody, indicating the expression of PS-receptors on the surface of macrophages. When macrophages were incubated with PS-liposomes, the fluorescence intensity decreased about 50%, but no change was observed in PC-liposomes, suggesting that PS-receptor is one of candidates for the binding site of PS-liposomes to macrophage. Macrophages express several receptors such as CD36, CD68, CD14, integrins which have an affinity to PS-liposomes, thus the ability of PS-liposomes to inhibit the binding of anti-PS-receptor antibody might be attenuated, and did not show the complete inhibition. Furthermore, this incomplete inhibition may come from the difference in the binding site of PS-liposome and the epitope of PS-receptor.

Suppression of NO Production by Anti-PS-Receptor Antibody To clarify whether PS-receptors contribute to the inhibitory effect of PS-liposomes on NO production, we examined the effects of antibody for the PS-receptor on the production of NO. As shown in Fig. 2, the production of NO stimulated by LPS was inhibited by the addition of the anti-PS-receptor antibody in a dose-dependent manner. No inhibition was observed when macrophages were treated with control antibody. The finding was compatible with the results in Fig. 1 that the binding of anti-PS-receptor antibody to macrophages was only inhibited in the presence of PS-liposomes. Taking these findings into consideration, the PS-receptor might contribute to the inhibitory effect of PS-liposomes on the production of NO in macrophages stimulated with LPS.

Activation of ERK and Induction of TGF-β by Anti-PS-Receptor Antibody TGF-β is a multifunctional cytokine that regulates numerous physiological processes, including cell growth, differentiation, apoptosis, adhesion, and the synthesis of extracellular matrix proteins.³⁰,³¹ Although TGF-β has been also recognized as an important immunoregulatory molecule, immunological responses of TGF-...
were often contradictory, and downregulation of the production of proinflammatory cytokines was reported.\textsuperscript{26,32} We previously reported that mouse peritoneal macrophages were induced to secrete TGF-\(\beta\) following PS liposome treatment, and TGF-\(\beta\) was intimately concerned with the inhibition of NO production by macrophages stimulated with LPS.\textsuperscript{19} Furthermore, the involvement of ERK, a MAP kinase, in the production of TGF-\(\beta\) by PS-liposomes was suggested.\textsuperscript{19} To clarify whether TGF-\(\beta\) production required the activation of ERK through the PS-receptor in mouse peritoneal macrophages, we first evaluated ERK activation by Western blotting following the treatment of macrophages with anti-PS-receptor antibody. As shown in Fig. 3A, treatment with the anti-PS-receptor antibody led to the activation of ERK MAP kinase after 15 min. Subsequently, we examined the changes in TGF-\(\beta\) mRNA and protein levels following treatment with the anti-PS-receptor antibody by RT-PCR and ELISA, respectively. Total RNA was extracted from macrophages treated with the antibody for 9 h, and then subjected to RT-PCR assay. TGF-\(\beta\) mRNA was expressed clearly, and the band completely disappeared when macrophages were treated with U0126, a specific inhibitor for MAP kinase kinase 1/2 (Fig. 3B). Furthermore, TGF-\(\beta\) protein was also produced by the anti-PS-receptor antibody and its production was suppressed by pretreatment with U0126 (Fig. 3C). These findings indicated that the TGF-\(\beta\) produced by macrophages was involved in ERK signaling via the PS-receptor.

We next examined whether the TGF-\(\beta\) produced through the anti-PS-receptor was involved in the inhibitory effects on NO production, we investigated the effects of anti-TGF-\(\beta\) antibody and U0126 on NO production by macrophages. As shown in Fig. 4, NO production inhibited by the anti-PS-receptor antibody was restored at the same levels by treatment with U0126 and anti-TGF-\(\beta\) antibody. This finding was the first evidence that the PS-receptor is involved in the regulation of NO production by mouse peritoneal macrophages stimulated with LPS, and TGF-\(\beta\) production via ERK activation is a prominent pathway that suppresses NO production. However, NO production was not restored completely, suggesting that TGF-\(\beta\) is one of factor and the other factor(s) may involved in the inhibition of NO production following PS-liposome treatment. Indeed, Vandivier et al. reported that several factors instead of TGF-\(\beta\) involved in downregulation of macrophages function following treatment with apoptotic cells.\textsuperscript{33}

Apoptosis is a critical process in tissue homeostasis and results in the immediate removal of dying cells by phagocytes such as macrophages and dendritic cells.\textsuperscript{34,35} This process should prevent inflammation and autoimmune responses against the intracellular antigens that can be released from apoptotic cells.\textsuperscript{36} Although there are a variety of mechanisms by which macrophages could phagocytose apoptotic cells, the best known molecule involved is PS.\textsuperscript{37} The PS-spe-
The specific receptor has been cloned and found to contribute to the phagocytosis of apoptotic cells. Furthermore, Huynh et al. reported that TGF-β expression was induced through the PS-receptor. However, it remains unclear how TGF-β is induced following the interaction of the PS-receptor with apoptotic cells. We made the interesting finding that the activation of ERK is a prominent pathway for the production of TGF-β through the PS-receptor.

In conclusion, the expression of PS-receptors on the surface of macrophages was confirmed by flow cytometry using an anti-PS-receptor antibody, and the binding of the antibody was specifically inhibited by PS-liposomes. The antibody also inhibited the production of NO by macrophages stimulated with LPS. ERK was activated when macrophages were treated with this antibody. Furthermore, the antibody led to an increase in the production of TGF-β. However, the production of TGF-β was largely inhibited by U0126, a specific inhibitor for ERK. With the addition of a TGF-β neutralizing antibody and U0126, the inhibitory effect of the anti-PS-receptor antibody on NO production was restored. These findings suggested that TGF-β is one of factors produced by PS-liposomes that suppresses production of NO in macrophages, and the ERK signaling pathway via the PS-receptor is intimately involved in TGF-β production by macrophages.

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